Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 43, Issue of October 27, pp. 33197–33200, 2000

Role of Sterols in Modulating the Human μ-Opioid Receptor Function in *Saccharomyces cerevisiae**

Received for publication, August 24, 2000, and in revised form, September 1, 2000
Published, JBC Papers in Press, September 5, 2000,
DOI 10.1074/jbc.C000576200

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This study provides evidence that the differences in membrane composition found from one cell type to another can represent a limiting factor to recovering the functionality of transmembrane proteins when expressed in heterologous systems. Restoring the properties of the human μ-opioid receptor in yeast (*Saccharomyces cerevisiae*), similar to those observed in native cells, was achieved by replacing ergosterol from yeast by cholesterol, which is normally found in mammalian plasma membranes. The results suggest that these two sterols have opposite effects with respect to the ligand binding function of the receptor. Ergosterol was found to constrain the μ-opioid receptor in an inactive state in yeast plasma membranes and cannot replace cholesterol in activating it. These data differ from previous works dealing with the function of related G-protein-coupled receptors (GPCR) in ergosterol-enriched membranes. This suggests that structural requirements of GPCR with respect to their modulation by lipid components differ from one protein to another. As a consequence, we assume that the presence of appropriate lipids around transmembrane proteins determines their function. This highlights the functional significance of lateral heterogeneities of membrane components within biological membranes.

To perform functional and structural studies of mammalian G-protein-coupled receptors (GPCR),[1] heterologous expression has long been a tool of choice (1). Unfortunately, although exceptions have been reported (2), these recombinant GPCR often fail to retain pharmacological properties similar to their native counterpart. Previous studies from our laboratory have focused on the expression of the human μ-opioid receptor in *Saccharomyces cerevisiae* (3). We found that the affinities of the antagonists were in the same range with yeast spheroplasts as in reference tissues. However those of agonists were shown to be lower. Assuming a lack of effective receptor coupling to endogenous G-protein α-subunits (*i.e.* GPA1 proteins (4, 5)), we were able to restore agonist binding upon addition of purified mammalian G-proteins (3). However, this approach requires high G-protein to receptor molar ratios and is not compatible with *in vivo* exploration of receptor functionality in heterologous systems. Finally, recent co-expressions of yeast/mammalian G-protein α-subunit chimeras with mammalian receptors have provided an alternative means to increase functional coupling (2, 6). According to this study and to restore human μ-opioid receptor activity in *S. cerevisiae*, we have co-expressed a $G\alpha_{i2}$-GPA1 chimera protein with the receptor. Unfortunately, as further shown below, this approach failed to restore the ligand binding function of the μ-opioid receptor.

Several investigations have suggested the importance of lipid membrane composition and organization in determining the activity of transmembrane proteins (7–11). Reports also emphasized the large diversity of lipid species found from one cell type to another (12, 13). Membrane composition and organization of heterologous systems different to those present in native cells may thus be the limiting factor on the recovery of fully functional recombinant transmembrane proteins. In this study, we then investigated an unreported way of restoring human μ-opioid receptor activity in *S. cerevisiae* by selectively modifying its lipid membrane content. To be precise, our approach deals with the replacement of ergosterol, which is specifically found in the plasma membranes of yeasts (14), by cholesterol, which is normally found in the plasma membranes of mammalian cells (12). The results obtained are in agreement with distinct roles of both sterols in determining the ligand binding function of the human μ-opioid receptor in *S. cerevisiae*.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The construction of the plasmid pEMR516 μ-OR carrying the cDNA coding for the human μ-opioid receptor is described in Ref. 3. Transcription of this gene is under the control of the GRAP1 promoter regulated by galactose. The chimeric Gα protein expression plasmid pLP82 was provided by M. H. Pausch (2). The strain LY296 (MATa, gpa1 Δhis G, far1Δ*LYS2*, sst2Δ *ADE2*, *FUS1-HIS3*, trp1, leu2, ura3, his3) used in this study is equivalent to the strain LY252 previously described (2) and was kindly provided by M. H. Pausch.

Yeast Culture, Induction Conditions, and Membrane Preparations—The spheroplasts and crude membranes are prepared as described in Ref. 3.

Replacement of the Sterol Content of Yeast Plasma Membranes—Spheroplast membranes (1.2 mg of proteins/ml) were incubated with 20 mM methyl-β-cyclodextrin (MBCD, Sigma) either loaded or not with cholesterol at a 1/18 cholesterol to cyclodextrins molar ratio in 50 mM Tris/HCl, pH 7.5. The buffer was supplemented with protease inhibitor mixture tablets (Roche Molecular Biochemicals) to prevent degradation of proteins. Shaking was performed for 30 min at room temperature. After centrifugation (35 min, 100,000 × *g*), the pellets were washed once with Tris-based buffer and then resuspended in 50 mM Tris/HCl, pH 7.5, 1 mM EDTA. Treated membranes were stored at −80 °C before use.

Biochemical Titrations—Protein content of spheroplast membranes was determined according to the Lowry procedure with bovine serum albumin as the standard. Total lipids were extracted from membranes following the method of Bligh and Dyer. The amount of phospholipids was estimated determining the phosphorus content according to Eaton and Dennis. Sterol content was determined using a colorimetric method based on the oxidation of the hydroxyl group at the carbon atom 3 in the β-position (Roche Molecular Biochemicals). Therefore, both ergosterol

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[1] The abbreviations used are: GPCR, G-protein-coupled receptors; MBCD, methyl-β-cyclodextrin; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5] enkephalin; DPN, diprenorphine; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; DPH, 1,6-diphenyl-1,3,5-hexatriene.

TABLE I

Binding affinities of modified yeast membranes expressing the human μ-opioid receptor and transformed (α_{i2}-GPA1 mutant strain) or not (GPA1⁻ mutant strain) with pLP82 containing $G\alpha_{i2}$-GPA1 cDNA

Corresponding dissociation constants (K_d) for [^{3}H]DAMGO and [^{3}H]DPN on untreated, ergosterol-depleted (20 mM MBCD) and cholesterol-loaded membranes (20 mM MBCD + cholesterol) determined as described in the legend to Fig. 2 and under "Experimental Procedures." Results are the mean results for three independent determinations performed in duplicate with corresponding S.D. ND, not detectable.

Yeast phenotype	Ligand	K_d		
		Untreated yeast membranes	Ergosterol depleted yeast membranes	Cholesterol loaded yeast membranes
			nM	
α_{i2}-*GPA1*	[^{3}H]DAMGO	ND	5.5 ± 1.5	3.2 ± 0.6
α_{i2}-*GPA1*	[^{3}H]Diprenorphine	1.0 ± 0.1	1.5 ± 0.2	0.7 ± 0.3
GPA1⁻	[^{3}H]DAMGO	ND	ND	5.5 ± 1.5
GPA1⁻	[^{3}H]Diprenorphine	3.8 ± 0.3	1.1 ± 0.2	0.3 ± 0.1

and cholesterol react in the assay. This explains the additional use of gas chromatography to quantify the relative amounts of both sterols when they co-exist within cholesterol-loaded yeast membranes (see Fig. 1).

Saturation Binding Experiments—Saturation experiments were performed on membrane aliquots (50–150 μg of protein) in 0.5 ml final volume of 50 mM Tris/HCl, pH 7.5, using 10 concentrations (0.2–20 nM) of [^{3}H][D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin ([^{3}H]DAMGO) or (0.2–6 nM) [^{3}H]diprenorphine ([^{3}H]DPN). Unlabeled ligands were used to determine nonspecific binding. Following a 1-h incubation period at 25 °C, free ligand was removed by filtration onto Whatman GF/B filters, and bound radioactivity was measured. Data were analyzed with the PRISM program.

RESULTS

Co-expression of a $G\alpha_{i2}$-GPA1 Chimera Protein with the Human μ-Opioid Receptor in S. cerevisiae—In our attempt to restore the human μ-opioid ligand binding function in *S. cerevisiae* (3), co-expression of a $G\alpha_{i2}$-GPA1 chimera protein with the receptor was performed as a first approach (see "Experimental Procedures"). This method was previously found to promote functional coupling of G-proteins to receptors, thereby leading to high affinity binding of agonists (2, 6). The results obtained following saturation binding experiments of the antagonist [^{3}H]DPN and the full agonist [^{3}H]DAMGO are shown in Fig. 2 and Table I. As shown previously (3), a high affinity binding for [^{3}H]DPN was observed (K_d = 1 nM). In contrast, we were not able to detect specific and saturable binding for [^{3}H]DAMGO. Accordingly, we assumed that G-protein/receptor coupling was not the main (at least the sole) limiting step to restore the human μ-opioid receptor ligand binding function in yeast.

Modifying the Sterol Content of μ/$G\alpha_{i2}$-GPA1-expressing Yeast Membranes—Replacement of ergosterol from *S. cerevisiae* plasma membranes by cholesterol was achieved using MBCD previously described as a sterol-carrying agent (10, 15). Spheroplast membranes co-expressing the human μ-opioid receptor and the $G\alpha_{i2}$-GPA1 chimera protein were incubated with 20 mM MBCD either loaded or not with cholesterol, thus leading to cholesterol complementation or ergosterol depletion, respectively. Efficiency of treatments, both determined by a 3βOH-sterol oxidase based method and by gas chromatography is presented in Fig. 1. The protein to phospholipid ratios measured following MBCD-mediated treatments of yeast membranes were unaltered, thus demonstrating the specificity of MBCD in carrying sterols (not shown). Binding parameters for [^{3}H]DPN and [^{3}H]DAMGO on treated membranes are shown in Fig. 2 and Table I. With respect to the antagonist [^{3}H]DPN, a 10-fold increase in binding (B_{max}) was found upon removal of ergosterol, still with high affinity properties. Similar patterns were obtained upon addition of cholesterol. Strikingly, restoration of saturable binding was seen on ergosterol-depleted membranes, although with slightly lower affinities (K_d = 5.5 nM) in comparison with those reported in mammalian-expressing systems (16). Loading untreated membranes with cholesterol yielded a 2-fold additional binding of the agonist as well as higher affinity (K_d = 3.2 nM).

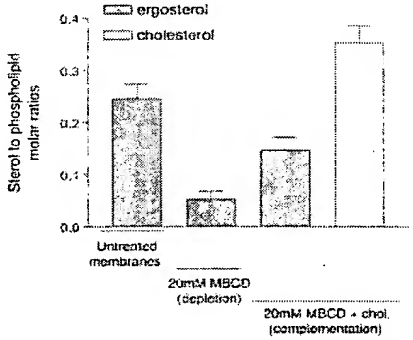

FIG. 1. **Methyl-β-cyclodextrin-mediated alterations of sterol amount in spheroplast membranes expressing the human μ-opioid receptor.** Incubations of yeast membranes with 20 mM MBCD either loaded (cholesterol complementation) or not (ergosterol depletion) with cholesterol at a 1/18 cholesterol to cyclodextrins molar ratio were performed for 30 min at room temperature in 50 mM Tris/HCl under continuous stirring. After centrifugation and a washing step to remove residual cyclodextrins, the membrane pellets were resuspended in 50 mM Tris/HCl, 1 mM EDTA. Quantification of phospholipid contents was carried out according to the procedure described under "Experimental Procedures." Both ergosterol (*filled bars*) and cholesterol (*open bars*) contents were determined using a 3βOH-sterol oxidase-based assay kit and by gas chromatography. Results are the mean results for four determinations carried out on both $G\alpha_{i2}$/*GPA1* and *GPA1*-deleted mutant strains with corresponding S.D.

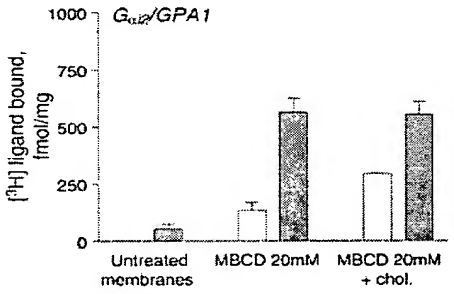

FIG. 2. **Binding capacities of modified yeast membranes from ΔGPA1 mutant strains transformed with pEMR516 μ-OR AND pLP82 containing $G\alpha_{i2}$/GPA1 cDNA.** Extents of specific binding (B_{max}) for [^{3}H]DAMGO (*open bars*) and [^{3}H]DPN (*filled bars*) were determined on untreated ergosterol-depleted (*MBCD 20 mM*), and cholesterol-loaded (*MBCD 20 mM + chol.*) membranes carrying out saturation binding experiments. Yeast membranes were incubated for 1 h at 25 °C in 0.5 ml final volume of 50 mM Tris-HCl, pH 7.5, containing 10 concentrations of radioligands ranging from 0.2 to 6 nM ([^{3}H]DPN) or 0.2 to 20 nM ([^{3}H]DAMGO). After filtration to remove free ligand, bound radioactivity was measured. Unlabeled ligands were used to determine nonspecific binding. Results are the mean results for three independent determinations performed in duplicate with corresponding S.D.

Considered altogether, the data presented here suggest that the human μ-opioid receptor resides in a prevailing low affinity state in *S. cerevisiae* plasma membranes, irrespective of the antagonist or agonist nature of ligands used. The detrimental effects of yeast membrane composition with respect to μ-opioid receptor function is further emphasized by specific binding recovery upon either removal of ergosterol or loading of cholesterol. Thus, conversion from low affinity to high affinity sites is likely to take place following the above treatments.

Uncoupling G-proteins from the Human μ-Opioid Receptor in

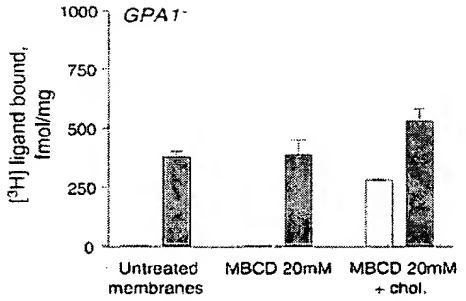

FIG. 3. **Binding capacities of modified yeast membranes from ΔGPA1 mutant strains transformed with pEMR516 μ-OR.** Extents of specific binding (B_{max}) for [^{3}H]DAMGO (*open bars*) and [^{3}H]DPN (*filled bars*) were determined on untreated, ergosterol-depleted (*MBCD 20 mM*) and cholesterol-loaded (*MBCD 20 mM* + chol.) membranes carrying out saturation binding experiments as described in the legend of Fig. 2. Results are the mean results for three independent determinations performed in duplicate with corresponding S.D.

Sterol-modifyed Yeast Membranes—Receptor coupling to heterotrimeric G-proteins is reported to increase the affinity of receptors for their agonists. To determine whether the recovery of high affinity binding of the agonist was related to G-protein coupling to the receptor, the above experiments were repeated on *GPA1*-deleted spheroplast membranes. Results are presented in Fig. 3 (B_{max}) and Table I (K_d). In contrast to what is obtained on μ/α_{i2}-*GPA1*-expressing spheroplast, the magnitude of binding (B_{max}) of the antagonist [^{3}H]DPN was little affected upon modulation of sterol content. However, a lower affinity (K_d = 3.8 nM) was measured on untreated membranes. Altering the sterol content of spheroplasts increases the affinity of receptors, either through removal of ergosterol (K_d = 1 nM) or by addition of cholesterol (K_d = 0.3 nM). Hence, both modifying sterol content procedures resulted in a stabilization of high affinity binding states for [^{3}H]DPN. In contrast, ergosterol depletion failed to restore specific binding for the agonist [^{3}H]DAMGO. Therefore, recovery of agonist binding upon removal of ergosterol on α_{i2}-*GPA1*-expressing spheroplast membranes probably also depends on G-protein/receptor coupling. Only cholesterol loading was found to restore binding of the agonist [^{3}H]DAMGO, although with a 2-fold lower affinity (K_d = 5.5 nM) than the one determined on α_{i2}-*GPA1*-expressing spheroplast membranes. This strongly suggests that cholesterol is able to stabilize a high affinity binding state of the μ-opioid receptor in yeast, irrespective of coupling to a G-protein. Furthermore, to prevent a possible coupling between the receptor and *GPA2*, another Gα-like protein also described in yeast (17), agonist binding was also performed with 100 μM Gpp(NH)p, a nonhydrolyzable GTP analog well known to uncouple the G-protein from the receptor. This did not alter agonist binding either (B_{max} = 300 fmol/mg, K_d = 4.5 nM).

DISCUSSION

The Pharmacological Properties of the Human μ-Opioid Receptor in S. cerevisiae Are Determined by the Lipid Composition of Yeast Plasma Membranes—Based on distinct pharmacological properties, three conformational states of human μ-opioid receptor have been identified. A model accounting for the results obtained including R_1, R_2, and R* as various states of the receptor is shown in Fig. 4. We postulate that a proportion of each state within biological membranes is mainly governed by lipid components (particularly sterols). R_1 is the major form of human μ-opioid receptor present in native yeast membranes. This conformational state exhibits low affinity for both agonist and antagonist. In contrast, in ergosterol-depleted membranes the R_2 conformational state binds antagonists with high affinity. The [R_1] *versus* [R_2] ratio (*i.e.* equilibrium *1*) strongly depends on the presence of ergosterol in the membranes as demonstrated by enhanced binding for [^{3}H]DPN upon its removal. These data suggest that ergosterol acts as an inhibitor of μ-opioid receptor binding function, constraining it in an inactive state R_1 when expressed in yeast plasma membranes. This is also supported by the fact that agonist binding is partly restored upon depletion of ergosterol. It is widely admitted that agonist binding on GPCR results in an activated R* conformation of the receptor, thereby promoting coupling to a G-protein and the formation of a stabilized ternary complex displaying high affinity for agonists (18). Indeed, lack of functional G-proteins is detrimental to agonist binding (18). In ergosterol-depleted membranes, G-proteins were required to observe agonist binding. This confirms that the R_2 state is not by itself able to retain agonists binding but needs stabilization by a G-protein of the agonist-induced shift to an activated R* state of the receptor. According to the law of mass action, the amount of G-proteins will affect the magnitude of agonist binding, *i.e.* the extent of receptor in the R* state, through the equilibrium constant K_G between coupled and uncoupled receptor states. This explains previous results from our laboratory showing recovery of agonist binding on native yeast membranes, where essentially the R_1 form is found, upon addition of an excess of purified mammalian G-proteins (3). However, whether the R* state observed under these conditions derives from R_2 or R_1 is not known, thus the proposal of equilibriums 2 and 3 leading to R* in our model. Finally, addition of cholesterol to the yeast membranes increases agonist binding, irrespective of the presence of G-proteins. This is similar to what is observed for constitutively active mutant receptors (18). Thus, cholesterol seems to constrain the μ-opioid receptor in an active R* state, while ergosterol constrains it in an inactive state R_1.

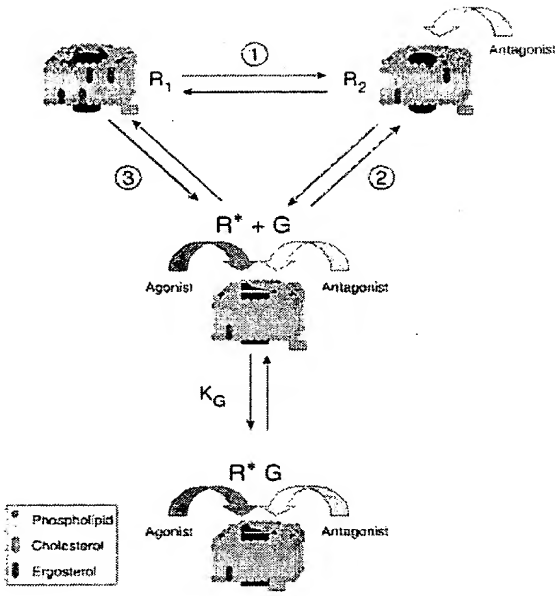

FIG. 4. **Depending on the membrane lipid composition, the human μ-opioid receptor can exist in equilibrium between three conformational states with distinct pharmacological properties.**

Accumulating evidence suggests that membrane sterols are unevenly distributed within the plane of membranes (19). Cholesterol-rich lipid rafts like platforms which recruit membrane proteins and support numerous cellular events in membrane traffic and signal transduction have been postulated (20). The

in vivo existence of rafts in mammalian cells has been demonstrated (21, 22). Works have provided evidence that ergosterol-mediated rafts also exist in yeast (23). We showed that the R_2 form of the μ-opioid receptor present in sterol-depleted membranes displays pharmacological properties distinct from those observed in a sterol-enriched membrane environment where the R_1 or R* states predominantly exist, depending on the presence of ergosterol or cholesterol in membranes (Fig. 4). Accordingly, distinct conformational and functional states of the μ-opioid receptor within biological membranes could be related to its distribution in sterol-rich and sterol-poor domains.

Works focused on the role that lipids play in assisting folding of membrane proteins, thereby leading to their proper conformational and structural organization (for review, see Ref. 24). As an example, the requirement of phosphatidylethanolamine as a molecular chaperone in determining assembly of lactose permease from *Escherichia coli* has been extensively studied (24, 25). Similarly, an alternative interpretation of our data includes partial misfolding of the human μ-opioid receptor when inserted in ergosterol-rich yeast plasma membranes. Then, modifying the lipid composition of yeast plasma membranes would result in restoring correct folding of the receptor and thus its functionality. However, whether or not ergosterol depletion and/or cholesterol complementation acts once the structure of the human μ-opioid receptor is restored is not known.

The Molecular Basis for the Modulation of Sterols on the Human μ-Opioid Receptor Function in S. cerevisiae—According to current literature (10, 26), the activity of transmembrane proteins such as the μ-opioid receptor function could be modulated through direct sterol-protein interactions or as a consequence of alterations of bulk physical properties of the lipid bilayer, following modifications of the sterol content. Experiments carried out both on model and natural membranes have emphasized similar roles for cholesterol and ergosterol in increasing ordering of acyl chains, thereby decreasing their fluidity state (10, 27). Thus, their very close capacity to alter bulk physical properties of membranes cannot account for the above mentioned distinct effects of cholesterol and ergosterol with respect to the μ-opioid receptor functionality. Furthermore, fluorescence polarization using DPH as membrane fluidity sensitive dye was found to be unaffected following the membrane treatments described above (data not shown). This strongly suggests that the modulation of μ-opioid receptor functions is unrelated to alterations of bulk properties of membranes. Thus, interactions between the μ-opioid receptor and lipids are likely to take place at the protein/lipid interface. One might assume that the minor structural differences between these sterols (27) are crucial in determining the μ-opioid conformation and function in a highly specific manner. Several investigations have dealt with the presence of adequate lipids near the annular environment of embedded proteins, thereby determining their structure and activity (7, 28). Similarly, an enrichment of sterol amount within the lipid layers surrounding the receptor might account for the observed alterations of its functionality. On the other hand, binding of lipids to specific recognition sites on transmembrane proteins to promote conformational changes as part of their function have been explored (10, 29). Quenching experiments of the intrinsic fluorescence of reconstituted proteins have provided suitable methods for probing lipid-protein interactions (28–30). Unfortunately, purification procedures for the μ-opioid receptor are still lacking.

To conclude, the above results emphasize the importance of specific lipid membrane composition and organization in maintaining membrane protein functions. We have shown that ergosterol, constraining the human μ-opioid receptor in an inactive state, cannot replace cholesterol that was found to activate the receptor. This strongly suggests that lipid composition of yeast plasma membranes was the main limiting factor in our attempt to fully restore the μ-opioid receptor functionality in *S. cerevisiae* (3). Accordingly, the addition of specific mammalian membrane lipids in yeast may provide new perspectives for the heterologous expression of fully functional mammalian receptors. Recent works dealt with the replacement of cholesterol by structurally modified sterols in mammalian plasma membranes to modulate the activity of two mammalian GPCR, the oxytocin receptor and the brain cholecystokinin receptor (10). In contrast to our results with the μ-opioid receptor, it was found that ergosterol was partially able to support the ligand binding function of the two related receptors. Other GPCR retain their properties when expressed in yeasts (2). In addition, yeast transmembrane proteins such as the α factor receptor involved in the mating response pathway in *S. cerevisiae* (31) are adapted to high levels of ergosterol. These data suggest that structural requirements displayed by GPCR with respect to their modulation by lipid components differ from one protein to another. As a direct consequence, this would imply the presence of suited lipids around transmembrane proteins to determine their function. This highlights the functional significance of lateral heterogeneities of membrane components within biological membranes.

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